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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
A01K 67/027, C12N 5/08

A1

(11) International Publication Number: WO 94/24853

(43) International Publication Date: 10 November 1994 (10.11.94)

(21) International Application Number: PCT/CA94/00249

(22) International Filing Date: 3 May 1994 (03.05.94)

(30) Priority Data:
055,105 3 May 1993 (03.05.93) US

(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, GE, HU, JP, KG, KP, KR, KZ, LK, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

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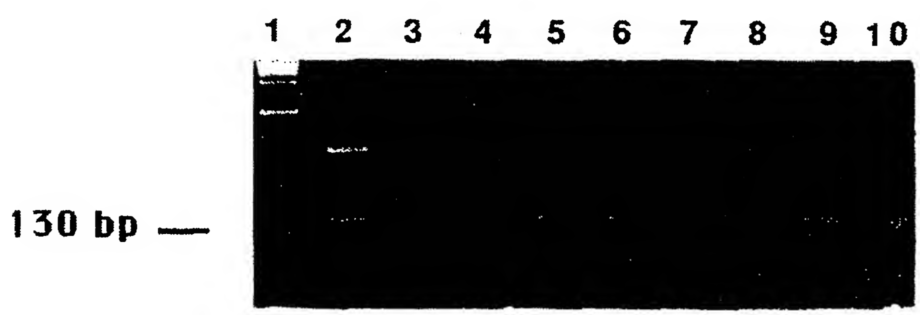
Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: ANIMAL MODEL FOR ENGRAFTMENT, PROLIFERATION AND DIFFERENTIATION OF HUMAN HEMATOPOIETIC STEM CELLS



(57) Abstract

Human/mammal immune chimeras can be generated that are genetically normal and exhibit sustained human immune cell proliferation and maturation in the absence of exogenous human growth factors.

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ANIMAL MODEL FOR ENGRAFTMENT, PROLIFERATION AND DIFFERENTIATION OF HUMAN HEMATOPOIETIC STEM CELLS

Background of the Invention

Numerous studies have demonstrated the importance of *in vivo* animal models in the study of mammalian organ systems, especially with respect to the immune systems. Unfortunately, researchers studying the human immune system have been without such a model. Recently, several groups have reported the engraftment of human bone marrow cells or human fetal liver cells into mice exhibiting severe combined immunodeficiency (SCID). Lapidot *et al., Science* 255:1137 (1992); Mosier *et al., Nature* 335:256 (1988); McCune *et al., Science* 241:1632 (1988). Another report used immunodeficient bg/nu/xid mice to achieve similar results. Kamel-Reid *et al., Science* 242:1706 (1988). None of these studies was able to establish long-term proliferation and differentiation of human tissues in the host. Additionally, transient differentiation was achieved only by the addition of exogenous human growth factors. Lethally-irradiated mice have also been used as recipients for human bone marrow cells. Lubin *et al., Science* 252:427 (1991). This study also failed to produce continued, normal human cell differentiation.

Hematopoiesis is a hierarchial process involving cells at various stages of differentiation and development. In the murine system, it is well-established that hematopoietic stem cells are capable of reconstituting the hematopoietic system of lethally-irradiated recipients. Jones *et al., Blood* 73(2):397 (1989). The most reliable assay for such activity is a transplantation assay demonstrating the reconstitution of primary and secondary recipients. Such an assay provides a valuable tool for the examination of the mouse.

immune system. However, because of the absence of a comparable model for humans, the understanding of human hematopoiesis is severely limited.

As mentioned above, there are reports of successful engraftment of human cells into immunodeficient mice. One of these studies, by Lapidot *et al.* (1992), used SCID mouse recipients for transplant of human bone marrow cells. When stimulated with combinations of erythropoietin (EPO) and human mast cell growth factor (hu-MFG), and/or PIXY321 (human IL-3 fusion protein), 76% of recipients showed engraftment of human cells in recipient bone marrow of 10 or more times that seen in animals receiving no growth factor treatment. Human tissue was of lymphoid, erythroid and myeloid character, indicating differentiation of transplanted tissue occurred. Without the addition of exogenous human growth factors, however, the relative amount of engraftment was low (0.01 to 1.0%). Moreover, it was unclear what effect extended discontinuation of growth factor treatment might have on subsequent stimulation. While this, and other previous studies represent important steps forward, they fall far short of a complete, functioning model of human hematopoiesis.

To date, however, no successful long-term engraftment, proliferation and differentiation of normal hematopoietic stem cells in a non-human mammal has been reported. As a result, no adequate animal model exists for the study of human hematopoiesis.

Summary of the Invention

It is, therefore, the object of the present invention to provide a closed, non-human model for the human hematopoietic system that is complete with respect to maintenance, proliferation and differentiation of human hematopoietic tissues.

Another object of the present invention is to provide a method by which non-human mammals, capable of supporting the maintenance, proliferation and

differentiation of human hematopoietic tissues without the addition exogenous factors, can be produced.

Another object of the present invention is to provide human tissue that is produced in a non-human mammal.

Another object of the present invention is to provide a method by which human tissue that is produced in a non-human mammal.

In satisfying the foregoing objects, there has been provided, in accordance with one aspect of the present invention a non-human, genetically-immunocompetent mammal, the hematopoietic system of which consists essentially of cells that are of human origin, wherein some non-lymphoid hematopoietic cells are syngeneic to said mammal.

There also is provided a process for producing the non-human mammal as described above comprising the steps of

(A) providing a non-human mammal in which immunologic genotype comports with the norm for the species of said mammal;

(B) exposing said mammal to a level of x- or gamma-radiation that is sufficient to destroy substantially all bone marrow of said mammal; then

(C) transplanting into said mammal syngeneic spleen colony cells and human cells comprising passaged bone marrow stromal cells.

There also is provided a non-human mammal that is the product of a process comprising the steps of

(A) providing a non-human mammal in which immunologic genotype comports with the norm for the species of said mammal;

(B) exposing said mammal to a level of x- or gamma-radiation that is sufficient to destroy substantially all bone marrow of said mammal; then

(C) transplanting into said mammal syngeneic spleen colony cells and human cells comprising passaged bone marrow stromal cells.

Brief Description of the Drawings .

Figure 1 is a photograph of an acrylamide gel depicting the PCR analysis of DNA isolated from individual multilineage colonies generated in the human from CFU-GEMM assay with marrow cells from reconstituted Balb/c mice.

Figure 2 shows an autoradiograph of a Southern blot analysis of DNA from 4 Balb/c mice reconstituted by CD34(+) cells (Group II).

Figure 3 shows micrographs of *in situ* hybridization of biotinylated human X-chromosome-→-satellite DNA.

Detailed Description of th Preferred Embodiments

This invention provides the first closed, long-term model for human hematopoiesis in a non-human mammal, and methods for production thereof. A closed model, for the purpose of this application, is defined such that the system of interest is capable of normal function without the addition of elements exogenous to the model organism. As a result of this capability, human hematopoietic systems can be studied more effectively, not only in general, but also in individual human patients. In addition, it permits the production of human tissues for diagnosis and treatment of human disease.

The present invention demonstrates that engraftment, proliferation and differentiation of human hematopoietic stem cells can be achieved in a non-human transfer recipient. Engraftment is detected by human cell-specific colony assay. Proliferation is confirmed by the presence of committed progenitors long after transplant in the colony assay. Differentiation is confirmed by the finding of

human cells of myeloid, erythroid and lymphoid nature. Active hematopoiesis is maintained without the addition of exogenous factors. And surprisingly, recipients of transplanted cells are not recognized as foreign by transferred human cells capable of such recognition.

Experiments conducted up to nine months after transfer of human cells show that both early and committed progenitor cells were maintained by recipients. Such cells could only be found in the presence of continued proliferation and maturation of the transplanted material. Therefore, these experiments, described in detail below, demonstrate the first long-term maintenance of human hematopoietic cells in significant numbers in a non-human recipient. It is also the first example of a complete, closed model of human hematopoiesis.

It is well-known that bone marrow-derived stromal cells provide a microenvironment able to support and regulate hematopoiesis in long-term bone marrow culture. Singer *et al.*, ADVANCES IN HAEMATOLOGY, Vol. 4, pp.1-34 (Hoffbrand, V., ed., Churchill Livingston, London, 1985); Dexter *et al.*, *J. Cell Physiol.* 82:461 (1977). Investigators have reported that stromal cells regulate hematopoiesis by providing cell-cell contact as well as by producing hematopoietic cytokines. Albertson *et al.*, *EMBO* 7:2801 (1988); Gualtieri *et al.*, *Exp. Hematol.* 15:883 (1987); Naparstek *et al.*, *J, Cell Physiol.* 126:407 (1986). As one might suspect, the lack of such factors in non-human recipients of human hematopoietic tissue markedly reduces or prevents the proliferation and differentiation of such tissues. Lapidot *et al.* (1992). In the present invention, this difficulty is avoided by the co-infusion of passaged stromal cells. Because these cells also successfully engraft, as reported by Wu and Keating, *Exp. Hematol.* 19:485 (1991), the factors necessary for proper development of human hematopoietic tissues are produced within the transfer recipient. This obviates the need for time-consuming and expensive addition of exogenous growth factors as reported elsewhere.

As will become evident, when transplanting human tissue into non-human hosts, it is highly desirable to use immunologically normal recipients. In this context, "immunologically normal" denotes an individual that displays immune system characteristics typical for the species to which the individual belongs. These characteristics would typically include, among others, functioning B-cells and T-cells as well as structural cell components, called cell surface antigens, which act as the immunologic signature for a particular organism.

Typically, the use of such immunologically normal recipients poses the following problem. The recipient's immune system, via its B- and T-cells, will identify the cell surface antigens of the engrafted tissue as foreign. This recognition leads ultimately to an immune response against the tissue, resulting in destruction of non-engraftment. This response is known as host-versus-graft rejection.

One way to circumvent host-versus-graft rejection is to use immunologically compromised recipients. Such animals exhibit two general types of deficiency, genotypic and phenotypic. Some researchers have employed genotypically-immunodeficient mice in order to circumvent this problem. These animals have genetic defects which result in the inability to generate either humoral or cell-mediated responses and include SCID mice and bg/nu/xid mice. Kamel-Reid *et al.* (1988); Lapidot *et al.* (1992). Therefore, they are unable to react against engrafted tissue. As a general proposition, however, the use of such animals is severely limited by the availability of an appropriate, immune-deficient organism as a recipient. In addition, these animals require housing in sterile environments and/or constant prophylactic antibiotic treatment.

The second category of immunodificient recipients are those which are genetically capable of generating an immune response, yet have been phenotypically altered such that no response is seen. Typically, such phenotypically-immunodeficient recipients are generated by irradiation and this technique has been used extensively. See Jones *et al.* (1989). Such an approach is not without its difficulties, however. Irradiation sufficient to render the

recipient incapable of mounting a response to the engrafted tissue usually results in death of the recipient due to destruction of the hematopoietic system.

The present invention obviates the need to create and/or identify genetically-immunodificient organisms as tissue recipients because immunodeficiency is achieved via irradiation of the recipient organism, and is therefore, phenotypic. Thus, any non-human mammal may be a recipient of human cells, permitting selection of the most favorable recipient, depending on the particular phenomenon to be examined. In addition, by selecting recipient organisms capable of supporting quantitatively greater human cell growth, the potential for increased human tissue proliferation is enhanced. Such non-human mammals will include, but are not limited to, mice, rats, rabbits, cats, dogs, pigs, sheep and non-human primates including baboons and chimps. It will also be unnecessary to maintain special colonies of potential recipients under sterile conditions or antibiotic maintenance.

The present invention also obviates the difficulties associated with irradiation by providing a replacement hematopoietic system following the destruction of the resident one. Specifically, by employing a set of human bone marrow stem cells capable of directing proliferation and differentiation of stem cells, transfer of a stable and functioning hematopoietic system is accomplished. Thus, animals that are successfully engrafted can survive the normally lethal radiation treatment.

A second problem results when, as in the present invention, the engrafted tissues are themselves capable of mounting an immune response. Such a response is called graft-versus-host phenomenon. This effect is mediated by T-cells within the transferred cell population. Only through elaborate, expensive, and time-consuming procedures can T-cells be eliminated from the transferred cell population. Previous studies of human cells transferred into non-human hosts have not directly addressed this issue. In fact, it is unclear whether graft-versus-host reactions actually occur in SCID mice. Lapidot *et al.* (1992).

The present invention, by way of contrast, employs a system where one expects graft-versus-host reactions. Yet here, there is a surprising lack of immune response by the grafted human cells against the host. This suggests a fundamental difference in human T-cell development and/or function following transfer into a non-human host. Regardless of the mechanism, the absence of graft-versus-host reactions in the present invention allows the use of normal human tissues without concern for the presence of T-cell activity.

Another important aspect of the present invention is the co-infusion of human cells with syngeneic non-lymphoid spleen colony cells. These cells are known to have profound effects on hematopoietic reconstitution and, to a limited extent, exhibit hematopoietic potential. Kitamura *et al., Nature* 291:159 (1981).

In addition to providing the first general animal model for human hematopoiesis, the present invention permits the study of the hematopoietic system of a particular patient. Thus, abnormal hematopoietic systems can be examined on an individual basis and compared to model systems derived from normal patients. The medical conditions which could be examined in this manner might include, but are not limited to acute and chronic leukemias of myeloid, lymphoid or multilineage cell origin, the myelodysplastic syndromes, myeloproliferative disorders, aplastic anemia, disorders involving deficiencies of single hematopoietic lineages such as pure red cell aplasia, thrombocytopenia or neutropenia and AIDS. As a result, subtle differences in both the pathology and responsiveness to treatment in a given patient can be examined outside that patient's body. The benefits of having such "custom-made" experimental vessels at the organismal level are apparent.

The present invention also provides for the use of non-human recipient organisms as "factories" for human tissues. One previous limitation in human biological and medical research has been the lack of human tissues on which to conduct research. If appropriate tissues are not available in a timely fashion, or in sufficient quantities, the ability of the investigator to conduct meaningful experiments can be impaired.

However, if small amounts of human tissue could be propagated outside the human body, the potential for producing relatively large quantities of human tissues could be realized. There have been two general approaches employed to solve this problem. The first, tissue culture of human cells *in vitro*, is generally limited by the mortality of cells outside the human body. The exception to this rule is the propagation of transformed cells. These cells, however, are generally not representative of normal cells and are only available on a fortuitous basis.

The other method used to produce human tissues is by grafting into non-human hosts. Yet this technology is limited by the immunologic reactions, by and against grafted tissue, described more fully above. One way to circumvent this phenomenon is to use hosts which are unable to mount an immune response to grafted tissues, such as genetically or phenotypically immunodeficient recipients. As mentioned, the use of genetically immunodeficient organisms is less than ideal due to the total immunodeficiency of the organism and the limitation as to the size and type of animal that may be used. The irradiated recipient, while circumventing these problems, faces the alternative difficulty of surviving radiation sufficient to knock out its immune function.

By practicing the present invention, one skilled in the art can overcome all the difficulties described above in the production of human tissue. Employing irradiated animals, one may select an appropriate host exhibiting any given desirable biologic characteristic. Further, repopulating the irradiated recipient with bone marrow stem cells results in the reestablishment of both immunocompetency and hematopoiesis in the host organism, thus obviating health concerns. Thereafter, hematopoietic cells, or any other co-infused, non-hematopoietic human tissues which engraft and proliferate, can be harvested. Such non-hematopoietic cells might include, but are not limited to liver, pancreas, brain, intestine, bone and cartilage. In many cases, the irradiation and engraftment may be performed on fetuses after removal from the womb, followed by reimplantation. In this way, the recipient organism (i.e., the fetus) can be protected by the mother's immune system prior to the establishment of the

transferred human immune system. In addition, it may be possible to replace entire recipient organs or organ systems with tissue derived from a single human patient, effectively creating an "ersatz" human in the non-human recipient.

Due to the complexity of the human system, it was found more instructive to initially use enriched or purified cell populations to study hematopoietic stem cells. Cell purification can be based upon the presence of the cell surface antigens mentioned previously. The CD34 antigen is one of the best-characterized human hematopoietic stem cell antigens, being expressed in 1% - 3% of normal human bone marrow cells. Bone marrow cells that express CD34 include colony-forming cells of all lineages, as well as their precursors. Experiments show that the CD34+ marrow cell fraction is enriched for a variety of primitive, multipotent, and committed progenitors (Civin *et al., J. Immunol.* 133:157 (1984); Saeland *et al., Blood* 72:1580 (1988)) which, in the presence of appropriate stimuli, can differentiate into myeloid or erythroid colonies *in vitro* and are capable of reconstituting normal marrow function in lethally irradiated primates. Berenson et *al., J. Clin. Invest.* 81:951 (1988).

In one version of the present invention, lethally-irradiated mice are co-infused with syngeneic mouse spleen colony cells, human marrow cells enriched for the CD34+ fraction, and passaged human bone marrow stromal cells. Surviving transplant recipients are screened by PCR and found to contain human DNA sequences. Examination of transplant recipient's bone marrow cells four months after engraftment detects from 11.9 to 68.3 precent human hematopoietic progenitors using a human hematopoietic colony assay. In contrast, engraftment of human hematopoietic progenitors in transplant recipients who do not receive co-infused human marrow stromal cells is 2.9 precent or less. Confirmation of human origin of hematopoietic progenitors is established by analysis of individual colonies using PCR amplification of human X-chromosome specific sequences and corroborated by *in situ* hybridization of marrow cells with a human X-chromosome specific biotinylated probe. Southern blot analysis of DNA extracted from the spleen, thymus and bone marrow of the transplanted

animals indicates that human cells were evenly distributed in these tissues. Transplant recipients tested nine months after co-infusion show significant numbers of mature human granulocytes, demonstrating sustained hematopoiesis of human immune cells.

The preceding paragraph underscores the importance of the inclusion of stromal cells with transplanted tissues. When transplanting non-hematopoietic tissues, other stromal cells or the relevant analogue can be used. Liver stroma (K①pfer cells, *etc.)* would be used when transplanting liver tissue, pancreatic stroma would be used when transplanting islet cells and microglial cells would be used when transplanting brain tissue.

The finding that stromal cells play an important role in facilitating engraftment of foreign tissues comports with other recent findings. For example, stromal cells from malignant tissues have been shown to mediate attachment, metastasis and growth in Hodgkin's and non-Hodgkin's lymphoma, breast cancer and prostate cancer.

Furthermore, human marrow stromal cells can be readily transfected with foreign genes using physical methods. Therefore, genetically modified stroma could be used to modify the recipient further. Keating *et al., Exp. Hematol.* 18:99 (1990); Matthews *et al., Exp. Hematol.* in press (1993).

In light of the preceding description, one skilled in the art can use the present invention to its fullest extent. The following examples therefore are to be construed as illustrative only and not limiting in relation to the remainder of the disclosure.

Example 1: <u>Human CD34+ Cell Isolation</u>

Cells from normal human bone marrow bearing the CD34 antigen are isolated using an enrichment method which gave 99% pure CD34+ cells, according to an immunofluorescent assay as follows. Light-density mononuclear cells are isolated by Ficoll-Hypaque gradient separation at a density of 1.077 g/ml. Cells bearing the CD34 antigen are isolated from a non-adherent

mononuclear fraction by positive selection using indirect immune panning with an anti-CD34 monoclonal antibody (HPCA-1; Becton-Dickinson, Mountain View, CA) as reported by Saeland *et al., Blood* 72:1580 (1988). A second purification step is performed using immunomagnetic beads. The CD34+ cells are resuspended at 10^7 cells/ml with immunomagnetic beads (10^7 beads/ml) coated with anti-mouse immunoglobulins for 30 minutes (Dynal Inc.). The beads are removed using a magnet, and the CD34+ cells were recovered in suspension. In all experiments, the isolated cells are 95% to 99% CD34+, as judged by staining with the anti-CD34 MoAb.

Example 2: <u>CFU-S Assay</u>

For co-infusion experiments with human cells, mouse spleen colonies are induced by the intravenous injection of Balb/c BM cells ($1x10^5$/mouse) into irradiated Balb/c mice (900 cGy) as described by Till and McCulloch, *Rad. Res.* 14:213 (1961). On day 12, the nodules developed on the spleen surface are harvested and single cell suspensions are prepared.

Example 3: <u>Human Stromal Cell Culture</u>

For co-infusion experiments with CD34+ cells and mouse spleen cells, human bone marrow stromal cell cultures are generated as described by Keating *et al., Blood* 64(6):1159-1162 (1984) and Keating *et al., Exp. Hemtol.* 18:99-102 (1990). Fresh human bone marrow mononuclear cells are placed into a 25 cm^2 tissue culture flask containing 7 ml McCoy 5A medium supplemented with 10% horse serum and 10% fetal bovine serum and 10^{-6} M hydrocortisone. The culture is incubated at 37°C in a humidified atmosphere containing 5% CO_2 in air; once a week, half the culture medium and non-adherent cells is removed until the adherent layer became confluent. After two to three weeks, the adherent layer is removed by treatment with trypsin, recultured in the same medium, and passaged a total of 3-4 times.

Example 4: Transplantation

In order to investigate if human CD34+ cells can be engrafted into normal murine recipients, one million such CD34+- enriched cells, the equivalent of 10^8 bone marrow cells, were transplanted into lethally-irradiated BALB/c mice (Jackson Laboratory, Bar Harbor, ME) in each of two groups - Groups I and II. Animals in both groups were transplanted with syngeneic mouse spleen colony cells in the amount of 3×10^6 per mouse in order to ensure murine hematopoietic reconstitution of the irradiated animals, but only animals in Group II received passaged human stromal cells in the amount of 1×10^7 cells per mouse. A total of 26 mice were transplanted with CD34+ cells and syngeneic spleen colony cells, of which 12, constituting Group II, are also transplanted with human marrow stromal cells. Of the 26 mice transplanted, 15 survived for more than four months. Eight of the 26 mice died during the first month, while three died during the 3 to 4 months after transplantation.

Example 5: Polymerase Chain Reaction (PCR)

Four months after transplantation, peripheral blood of the recipients was collected and examined for the presence of human cells by polymerase chain reaction (PCR) analysis. Individual colonies were picked from culture dishes. After washing once with distilled water, the spleen colony cells were digested in 100 µl of buffer [containing 200 µg/ml proteinase K, 50 mmol./L Tris-chloride (pH 8.5), 1 mmol/L EDTA, and 0.5% Tween 20] at 56°C for 1 hour with shaking. After digestion, the samples were boiled for 10 minutes to inactive proteinase K. For amplification, 5 µl of the sample was subjected to PCR amplification using 2.5 units *Taq* enzyme (Boehringer Mannheim, FRG), 250 ng of each primer, and 100 µmol/L of each dNTP (Boehringer) in a final reaction volume of 100 µl buffer. For the amplification of the human X alphoid repeat sequence, the primers of the sense and antisense were: 5'-AATCATCAAATGGAGATTTG-3', 5'-GTTCAGCTCTGTGAGTGAAA-3', respectively (Witt *et al., Human Genetics*

82:271-274 (1989). Amplification was at 94°C for 30 seconds, 54°C for 30 seconds, and 72°C for 1.5 minutes for 30 cycles. Amplified products were electrophoresed on 2.5% agarose (FMS) and stained by ethidium bromide. As shown in Table I, recipients in both experimental groups (with or without human stromal cells) contained human cells.

Example 5: <u>Colony Assay</u>

In order to further characterize these human cells, single cell suspensions of the recipient bone marrow were plated and colony assays, optimized either for the growth of a human multilineage colony (CFU-GEMM) or mouse granulocyte-macrophage progenitors (CFU-GM), were performed:

Human CFU-GEMM. Semisold cultures in methylcellulose are produced according to a standard method (Keating and Toor, [reference]), and modified by plating 1 x 10^5 cells per tissue culture grade 35 Petri dish, in the presence of 10% human plasma, 10% fetal bovine serum, 1-4 units/ml erythropoietin, rhSCF (CytoMed, MA) and rhIL-3 (Amersham). Duplicate dishes are plated in each experiment after 12 days of incubation at 37°C in 5% CO_2 in air. The colonies are counted using an inverted phase-contrast microscope.

Murine CFU-GM. Bone marrow cells are gently dispersed into a single cell suspension in Iscove's Modified Dulbecco's Medium (IMDM) containing 10% fetal bovine serum. To measure granulocyte-macrophage colony-forming cells (CFU-GM), bone marrow cells (1 x 10^5) are cultured in 1 ml IMDM containing 0.3% Difco agar and IL-3 (as produced from an IL-3-producing cell line provided by G. Mills, Toronto). After incubation for 7 days at 37°C in 5% CO_2 in humidified air, granulocyte-macrophage colonies (CFU-GM) containing >50 cells are counted. All cultures were performed in duplicate.

Since murine IL-3 does not stimulate the growth of human hematopoietic progenitor cells, the differing culture conditions allow a determination of cross-stimulation to be made. The results, set forth in Table II, show that no cross-stimulation was observed. Table II also contains a summary of the information

gained from an analysis of colonies obtained from the marrow cells of the recipient mice.

In Group II, a large proportion of early myeloid progenitors were detected under culture conditions suitable to human hematopoietic progenitors. Comparing this result to the colonies detected under culture conditions suitable for murine progenitors, the ratio of human:mouse colonies varied from 11.9% to 68.3%. In terms of colony formation, results were similar to those observed with normal human marrow controls. In contrast, the Group I recipient mice which did not receive human stromal cells contained very few human hematopoietic progenitors detected with the granulocyte-macrophage colony assay examined after 12 days in culture. No committed erythroid progenitors (BFU-E) were detected in this group.

Some of the Group II recipients were followed for 9 months after transplantation. An analysis of human hematopoietic progenitors present in the bone marrow of these recipients using an *in vitro* colony assay is shown in Table III. Human committed progenitors (3.8% to 23%) were found in three of the four mice. The human origin of individual colonies was confirmed by PCR analysis. The sustained maintenance of committed human hematopoietic cells in the recipient mice suggests that the engrafted CD34+ cells developed in the bone marrow of recipients and showed sustained proliferation and differentiation.

Example 6: PCR Analysis of Individual <u>Hematopoietic Colonies</u>

In order to demonstrate that the colonies detected under CFU-GEMM culture conditions are indeed human hematopoietic cells, PCR is used to amplify and detect the human X chromosome α-satellite repeat in individual human multi-lineage colonies. PCR amplification of normal human DNA results in a 130 bp band (Witt and Erickson, *Human Genetics* 82:271 (1989)). PCR was performed essentially as described above. To measure background amplification in the PCR assay, 100 ng of DNA from Balb/c granulocyte-macrophage colonies is used as a negative control.

The results, shown in Figure 1, indicate that all the colonies generated under culture conditions suitable for animal or human hematopoietic cells contained the 130 bp specific human DNA product, while no PCR amplification product was seen in the negative controls. For example, for recipient #4, 10 colonies were isolated and individually analyzed (Table I); all 10 were positive for the human sequence.

Example 7: Isolation of Genomic DNA and Southern Blot Analysis

In order to examine the tissue distribution of human hematopoietic cells in transplant recipients, Southern blot analysis is performed. Standard procedures (Maniatis *et al.*) for the preparation of genomic DNA samples are used. Ten µg of genomic DNA is digested with the appropriate restriction enzyme and the electrophoresed through a 0.7% agarose gel. Following Southern transfer of Hybond-N (Amersham) nylon membranes and subsequent baking, membranes are placed in a bag containing phosphate buffer prehybridization solution containing 5X SCC, 0.45% skim milk power, 0.1% SDS, pH 7.2 Blots are hybridized overnight at 42°C using the same prehybridization solution containing 9% dextran sulfate. After hybridization, the blots are extensively washed in 2X SCC, 0.1% SDS for 20 min. at room temperature and in 0.1X SCC, 0.2% SDS for 10-30 min. at 65°C. The autoradiograph is exposed at -70°C using an intensifying screen.

A human Factor 1X probe (McGraw *et al., Proc. Nat'l. Acad. Sci. USA* 82(9): 2847-2851 (1985), linear and gel purified, was labelled (>6 x 10^8 cpm/ µg DNA) with ^{32}P using the random primer method. As can be seen in Figure 2, all four recipients examined contained human cell DNA in thymic, splenic, and marrow tissues.

Example 8: *In Situ* Hybridization

Fluorescent *in situ* hybridization has become an important technique for visualizing genetic material in fixed cells. A major advantage of this method is

that interphase human hematopoietic cells, including immature hematopoietic cells, can be distinguished from murine cells using a human-specific probe. The *in situ* hybridization method can be used to further confirm the presence of human hematopoietic cells in the transplant recipients. For *in situ* hybridization, bone marrow cells are incubated in 75 mM KCl for 15 min. at 37°C. The cells are spun down and fixed with two changes of methanol/acetic acid (3:1 v/v). Cells are centrifuged on cleaned slides, allowed to air dry overnight, and gradually dehydrated with ethanol. Before use, slides are treated with RNase A (100 μg/ml) in 2X SSC for one hour at 37°C, with proteinase K (0.1 μg/ml in 20 mM Tris-HCI, 2 mM $CaCl_2$, pH 7.4), for 7.5 min. at 37°C and are post-fixed with 4% paraformaldehyde for 10 min. dehydrated, and kept at room temperature until used. DNA is denatured by immersion of the slides in 70% formamide in 2X SSC, pH 7, for two minutes at 70°C. This is followed by immersion in ice-cold 70% ethanol, and by continued dehydration with ethanol.

The probe is denatured by heating the hybridization mixture, followed by quick cooling on ice, and added to slides. After a coverslip is added and sealed with rubber cement, the slides are incubated in a moist chamber for 12 to 16 hours at 37°C. After hybridization, the slides are washed in two changes of 50% formamide, 2X SSC and in three changes of 2X SSC at 40°C for twenty min. each.

For detection of hybridization, the slides are overlayed with 10 μl fluorescein-labelled avidin (Vector Laboratories) in 2X SSC plus 1% BSA. After incubation of 45 min. at R.T. in the dark, the slides are washed in two changes of 2X SSC, 1X SC and 0.5X SSC, for 5 min. each, and then counterstained with propidium iodide (PI, 0.5 μg/ml) in anti-fade solution.

The probe, a human X-chromosome ∝-satellite DNA (Oncor Inc.) that does not hybridize with murine DNA (Waye *et al., Nucleic Acids Res.* 13(8):2731-2734 (1985)) (20 ng/μl), was added to hybridization mixture which contained 50% formamide, 2X SSC, and 500 μg/ml of carrier salmon sperm DNA. The photographs in Figure 3 were taken on Kodak Ektachrom 1600 film

with a Zeiss fluorescence microscope. The hybridization results are depicted in Figure 3. These results indicate the presence of human hematopoietic cells in the bone marrow of murine transplant recipients and confirm results obtained with Southern blots of marrow DNA and PCR analysis of individual hematopoietic colonies.

These results are the first to show the presence of very early as well as terminally differentiated human hematopoietic cells. Because donor human cells were enriched for early hematopoietic progenitors and lacked terminally differentiated cells, the appearance of significant numbers of mature human granulocytes as well as the detection of human multi-lineage colonies in mice reconstituted nine months previously, indicates that human donor cells not only engrafted, but proliferated and differentiated *in vivo* as well.

Example 9: <u>Analysis of Bone Marrow Cells from Murine Recipients Investigated Nine Months after Transplantation of Human CD34+ Cells and Human Passaged Marrow Stromal Cells</u>

Cell sorting experiments can determine levels of murine and human lymphoid cells in long-term reconstituted recipients. Analysis with a FACScan instrument was performed using the following monclonal antibodies:

1. <u>goat•anti-human IgG Fc:</u> **human B cells**
(affinity purified F(ab^1) 2 preparation, mouse Ig adsorbed and FITC labelled)

2. <u>mouse•anti-human CD3:</u> **human T cells**
(phycoerythrin labeled IgG2a)

3. <u>goat•anti-mouse IgG:</u> **murine B cells**
(affinity purified F(ab^1) 2 human Ig adsorbed and P-PE labeled)

4. <u>hamster•anti-mouse CD3:</u> **murine T cells**
(FITC labeled IgG)

Three color sorting with biotin/streptavidin-PerCP labeled anti-CD45 (T200) antibody, recognizing human/murine and human nucleated hematopoietic cells,

was used. The frequency of cells was established as follows: human B cells - 9%; human T cells - 12%, murine B cells 2%; murine T cells - 3%.

Example 10: PCR Analysis of Recipient Bone Marrow Cells for Human and Murine T and B Cells Nine Months after Transplantation

Human/murine lymphoid subpopulations were sorted using monoclonal antibodies as described in Example 9. The sorted subpopulations were subjected to PCR analysis for specific human and murine T and B cell sequences. PCR analysis were performed according to our modification of standard methods. Wu and Keating, (1993).

The following primers were used to detect Ig mRNA (*i.e.,* B cell message):

sense - Igh-J primer recognizing, human and murine J regions

antisense - CH1 region of murine IgG, recognizing all IgG isotypes

antisense - CH2 region of human IgG, recognizing all IgG isotypes

The following primers were used to detect T cell receptor mRNA:

sense - TCR β-chain-J regions recognizing, human and murine J regions

antisense - CHI region of murine TCR β-chain, recognizing all murine TCR β-chain isotypes

antisense - CH2 region of human TCR β-chain, recognizing all human TCR β-chain isotypes

For each set of primers, amplification was seen, thus confirming the presence of both human and murine B and T cells.

Table I.
PCR amplification of human DNA from reconstituted Balb/c mice using human X-chromosome α repeat primers

	Peripheral blood	Thymus	Spleen	Bone Marrow	Colonies in human CFU-GEMM assay
GROUP I					
Mouse #1	+	+	+	+	1/1
#2	+	+	+	+	1/1
#4	+	+	+	+	1/2
Total:					3/4
GROUP II:					
Mouse #1	+	+	+	+	7/7
#2	+	+	+	+	10/10
#3	+	+	+	+	10/10
#4	+	+	+	+	10/10
#5	+	+	+	+	10/10
#6	+	+	+	+	4/4
Total:					51/51

Group I Lethally-irradiated Balb/c mice were injected with CD34+ cells and spleen colony cells.

Group II Lethally-irradiated Balb/c mice were injected with CD34+ cells, spleen colony cells, and human stromal cells.

Table II. Hematopoietic colonies from Balb/c bone marrow reconstituted with CD34+ cells

	NCC per femur	Mouse CFU-GM[a]		Human CFU-GEMM[a]		Human: Mouse ratio
	$1x10^6$	$1x10^5$ cells	per femur	$1x10^5$ cells	per femur	
GROUP I[b]						
mouse #1	6.3	78±6.0	4914±37.8	1±0.2	63±3.2	1.2%
#2	3.9	71±3.4	2769±22.6	0.5±0.2	18±1.2	0.6%
#3	5.8	47±2.4	2726±13.9	0	-	-
#4	8.2	69±3.4	5658±27.8	2±1.0	164±7.8	2.9%
#5	4.2	58±4.2	2436±17.6	0	-	-
GROUP II[c]						
mouse #1	12.2	22±3.2	2684±24.2	6±0.4	732±12.1	27.2%
#2	6.9	48±2.4	3312±34.8	22±1.4	1518±16.2	45.8%
#3	9.4	28±1.4	2632±22.4	16±2.4	1504±12.4	57.1%
#4	7.7	41±2.2	3157±34.4	28±3.2	2156±28.2	68.3%
#5	14.6	68±3.4	9928±42.3	16±3.7	1088±14.2	23.5%
#6	8.2	42±2.1	3444±26.6	5±1.2	410±8.4	11.9%
Control-1[d]	9.8	88±4.2	8624±35.8	0		
Control-2[e]		0		75±2.4		

a. Results are the mean±SE from duplicate.
b. Lethally-irradiated Balb/c mice were transplanted with CD34+cells and spleen cells.
c. Lethally-irradiated Balb/c mice were transplanted with CD34+ cells, spleen cells, and human stromal cells.
d. Normal Balb/c bone marrow cells were cultured for CFU-GM.
e. Normal human bone marrow cells were cultured for CFU-GEMM.

Table III. CFU-GEMM from the bone marrow of Balb/c mice reconstituted with CD34+ cells, mouse spleen cells, and human marrow stromal cells

	NCC per femur	Mouse CFU-GM[a]		Human CFU-GEMM[a]		Human: Mouse %	PCR (+) (by human x primer)
	1×10^6	1×10^5 cells	per femur	10×10^5 cells	per femur		
mouse[b] #1	1.3	72±3.2	936±9.6	11±2.2	143±8.2	14.5%	5/5
#2	1.1	65±1.4	715±10.2	2±1.4	22±2.2	3.8%	2/2
#3	0.5	78±2.6	390±6.3	4±1.4	20±2.1	5.1%	4/4
#4	2.1	65±1.8	1365±7.4	15±2.3	975±4.4	23.0%	10/10
Control-1[c]	8.9	84±3.2	7476±18.8	0			
Control-2[d]		0		75±4.1			

a. Results are the mean±SE from duplicate cultures.
b. Lethally-irradiated Balb/c mice, reconstituted by transplantation with CD34+cells, syngeneic mouse spleen cells, and human stromal cells, were viable.
c. Normal Balb/c bone marrow cells were cultured for CFU-GM.
d. Normal human bone marrow cells were cultured for CFU-GEMM.

CLAIMS

1. A non-human, genetically-immunocompetent mammal, the hematopoietic system of which consists essentially of cells that are of human origin, wherein some non-lymphoid hematopoietic cells are syngeneic to said mammal.

2. A non-human mammal according to claim 1, wherein said mammal maintains human, hematopoietic processes.

3. A non-human mammal according to claim 2, wherein said mammal also maintains human, non-hematopoietic tissues.

4. A non-human mammal according to claim 1, wherein said mammal is a mouse.

5. A process for producing the non-human mammal of claim 1 comprising the steps of

(A) providing a non-human mammal in which immunologic genotype comports with the norm for the species of said mammal;

(B) exposing said mammal to a level of x- or gamma-radiation that is sufficient to destroy substantially all bone marrow of said mammal; then

(C) transplanting into said mammal syngeneic spleen colony cells and human cells comprising passaged bone marrow stromal cells.

6. A process according to claim 5 wherein transplanted human cells consist essentially of hematopoietic cells.

7. A process according to claim 5 wherein transplanted human cells consist essentially of hematopoietic and non-hematopoietic cells.

8. A non-human mammal that is the product of a process comprising the steps of

(A) providing a non-human mammal in which immunologic genotype comports with the norm for the species of said mammal;

(B) exposing said mammal to a level of x- or gamma-radiation that is sufficient to destroy substantially all bone marrow of said mammal; then

(C) transplanting into said mammal syngeneic spleen colony cells and human cells comprising passaged bone marrow stromal cells.

9. A non-human mammal that is the product of a process according to claim 8 wherein transplanted human cells consist essentially of hematopoietic cells.

10. A non-human mammal that is the product of a process according to claim 8 wherein transplanted human cells consist essentially of hematopoietic and non-hematopoietic cells.

11. A non-human mammal according to claim 9 wherein transplanted human hematopoietic cells consist essentially of passaged bone marrow stromal cells and CD34+ cells.

12. A method for the production of human tissue in a non-human mammal according to claim 2 comprising the further step of (D) harvesting said human tissue from said non-human mammal.

13. A method for the production of human tissue in a non-human mammal according to claim 3 by harvesting said human tissue from said mammal.

14. A method for the production of human tissue according to claim 12 wherein said tissue is of lymphoid origin.

15. A method for the production of human tissue according to claim 12 wherein said tissue is of erythroid origin.

16. A method for the production of human tissue according to claim 12 wherein said tissue is of myeloid origin.

17. A method for the production of human tissue according to claim 14 wherein said tissue is not of lymphoid, erythroid or myeloid origin.

18. Human tissue that is the product of the process according to claim 9 by harvesting said tissue from said mammal.

19. Human tissue that is the product of the process according to claim 10 by harvesting said tissue from said mammal.

20. Human tissue according to claim 18 wherein said tissue is of lymphoid origin.

21. Human tissue according to claim 18 wherein said tissue is of erythroid origin.

22. Human tissue according to claim 18 wherein said tissue is of myeloid origin.

23. Human tissue according to claim 19 wherein said tissue is not of lymphoid, erythroid or myeloid origin.

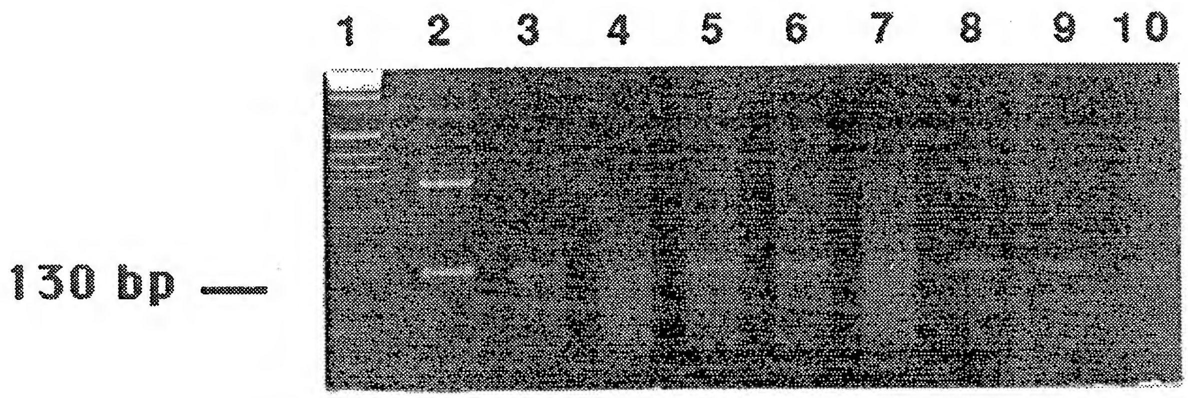
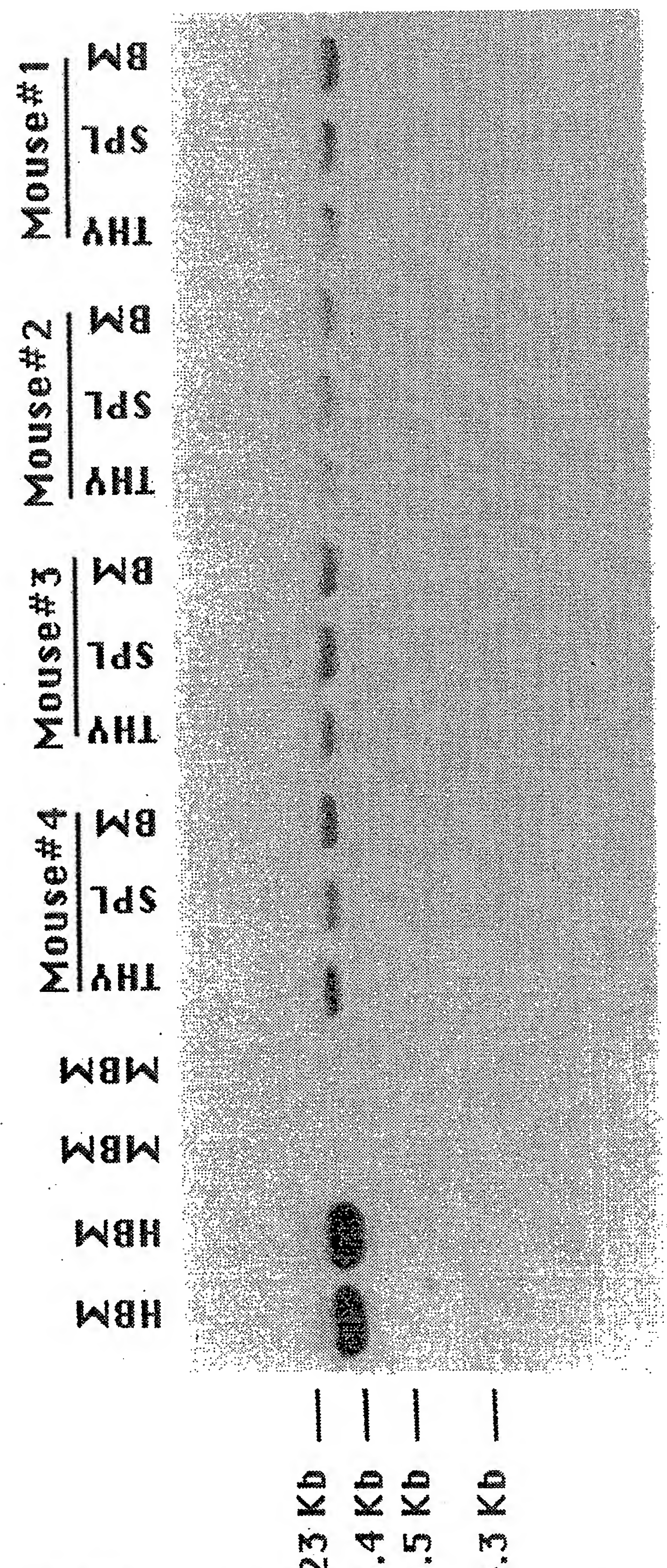


FIG.1.


Mouse#1
BM
SPL
THY
Mouse#2
BM
SPL
THY
Mouse#3
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THY
Mouse#4
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MBM
HBM
HBM
23 Kb
9.4 Kb
6.5 Kb
4.3 Kb

FIG. 2.

FIG 3A.

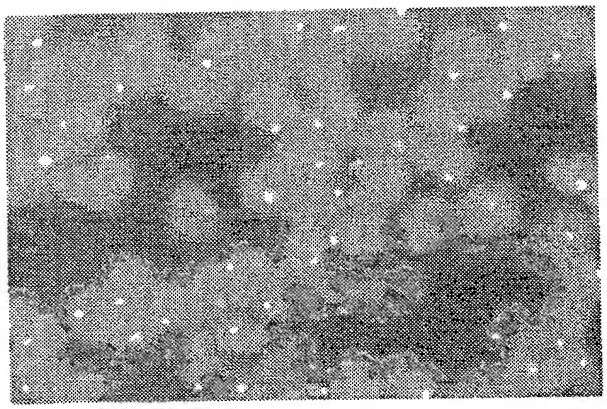


FIG 3B.

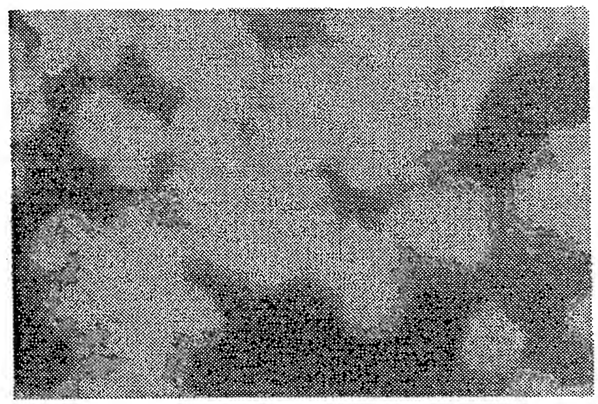


FIG. 3C.

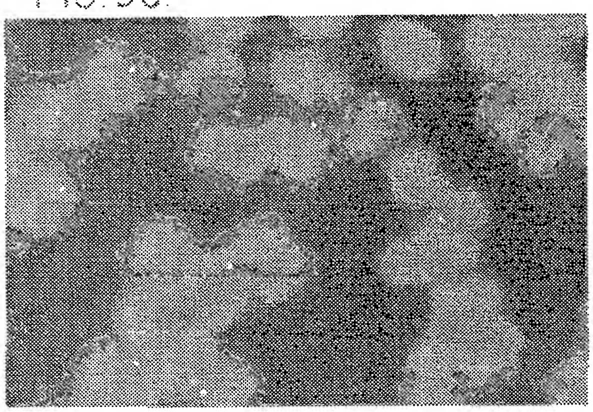
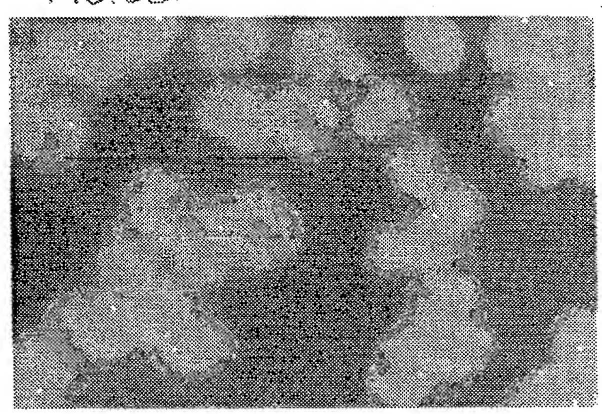


FIG. 3D.



INTERNATIONAL SEARCH REPORT

Intc .onal Application No
PCT/CA 94/00249

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 A01K67/027 C12N5/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 438 053 (YEDA RESEARCH DEVELOPMENT COMPANY) 24 July 1991 See claims 1-17; page 7, lines 6-15; examples	1,2,4
Y	See page 7, lines 5-15 ---	3,5-23
X	SCIENCE vol. 255 , 28 February 1992 , WASHINGTON pages 1137 - 1141 LAPIDOT T ET AL. 'Cytokine stimulation of multilineage hematopoiesis frpm immature h. cells engrafted in SCID mice' cited in the application	1,2,4
Y	See whole reference --- -/--	3,5-23

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
7 September 1994	1-4 -10- 1994

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SCIENCE vol. 242, no. 4885 , 23 December 1989 , WASHINGTON pages 1706 - 1709 KAMEL-REID S. ET AL. 'Engraftement of immune-deficient mice with human hematopoietic stem cells' cited in the application See whole reference	1,2,4
Y	See whole reference	3,5-23
Y	EXPERIMENTAL HEMATOLOGY vol. 19, no. 485 , 21 July 1991 page 585 WU D. ET AL. 'Engraftement of donor-derived bone marrow stromal cells' cited in the application See abstract	3,5-23
P,X	WO,A,93 09792 (IMMUNEX CORP.) 27 May 1993 See claims	1-4, 12-17

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INTERNATIONAL SEARCH REPORT

..formation on patent family members

Interr al Application No
PCT/CA 94/00249

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0438053	24-07-91	JP-A- 4267830	24-09-92
WO-A-9309792	27-05-93	AU-A- 3178193	15-06-93
		CA-A- 2122725	27-05-93